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Review

Role of oral fluids in DNA investigations

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A B S T R A C T

The assay of oral fluid (OF), a biofluid historically well-studied biochemically and physiologically, is a growing area of research with implications for basic and clinical purposes. In the last decade, it has gained considerable attention and lately, the use of OF has provided a substantial addition as an investigative tool in forensic and/or legal procedures. This article is an appraisal of various applications of OF sourced DNA in the field of forensic analysis. We have discussed the significance of different collection methods and their variations along with the application of specific analytical methods based on the condition of the sample. It is likely that the germaneness of OF assays will continue to expand thus providing a new instrument for investigation in criminal/legal proceedings.

1. Introduction

The detection and identification of body fluids in a criminal investigation are paramount inforensic science. Determining whether a body fluid is present and later identifying it allows further testing including DNA analysis.[1,2]

Despite a long history of study, OF's physiological importance and application in forensics is gaining recognition only in the recent times. It is a complex and dynamic biological fluid which is attaining prominence and an increased scientific interest as an alternative matrix in crime scene investigations, paternity testing and for age estimation.

Several fluids combine to constitute 'saliva'. Strictly speaking, 'saliva' is collected from a specific salivary gland and is free from other materials while 'oral fluid' or whole saliva constitutes secretions from several sources, including three pairs of major salivary glands, over 450–750 minor salivary glands [8%]), in addition to several constituents of non-salivary origin, such as gingival crevicular fluid (GCF), expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and bacterial products, viruses and fungi, desquamated epithelial cells, other cellular components, and food debris. However, in the scientific literature, the distinction between saliva and OF is rarely made.[3,4]

2. Salivary DNA

OF recovered at crime scenes from bite marks, cigarette butts, postage stamps, envelopes and other objects, is a known source of high molecular weight DNA.[5–7]

By tradition, high-quality genomic DNA is extracted from leukocytes in serum. However, due to the invasiveness and cost of obtaining, transporting, and processing blood samples, OF is now an important and reliable alternative for genetic research even when stored in different ways.[6,8] The genetic material in OF consists of a large variety of DNAs, RNAs, and proteins. Salivary DNAs represent (a) the hosting human genome, (b) the oral microbiota, and (c) the infecting DNA-viruses. Desquamated oral mucosal cells represent the main source of human DNA in the OF.[9] Apart from the host DNA, bacterial DNA is another marker of discriminative value within saliva deposited during a bite mark. There is evidence that each individual shelters a unique set of bacterial species that are identifiable through bacterial typing and protein profiles. Therefore, researchers suggest that the genotype identification of oral streptococci may further benefit bite mark analyses.[10,11]

Recent studies reveal that whole saliva samples with a minimum volume of 0.1 ml are sufficient to extract good quality and quantity DNA. This despite being subjected to processing for, storage, while shipping and when stored without refrigeration for (up to 5) days.[12,13] Thus, a combination of smaller sample and simple

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logistics make the OF an attractive biological sampling method for genotyping genetic polymorphisms.[7,14]

2.1. Forensic saliva tests

Prior to DNA extraction from a sample, identification for presence of body fluids as evidentiary materials is considered. Location and identification of body/oral fluids is achieved by using alternative light source technology (ALS) like UV light, high intensity quartz arc tubes, and fluorescence spectroscopy. The Lumatec® Superlight 400 (320–700 nm) can detect human saliva samples on different types, and colors of fabrics, both in darkness and daylight. Storage times of 3 and 5 weeks show no difference in the results.[15] Saliva can even be detected using a range of 415–490 nm with orange or red goggles.[2]

Once identified, the body fluid undergoes saliva presumptive tests. These tests detect either enzymatic activity of alpha amylase or the presence of saliva specific genes like statherin (STATH) and histatin 3 (HTN3). The Phadebas® test reagent, includes procion red amylopectin, available in tube and press tests which can detect saliva diluted up to 1:128. The Saligae® test (Abacus Diagnostics) is another colorimetric assay, wherein a solution changes into yellow colour with the addition of saliva extract and is also available as a spray.[2]

Juusola and Ballantyne (2005) proposed Real time Polymerase Chain Reaction (RT-PCR) method for detection of mRNA markers with saliva specific expression i.e. statherin (STATH) and histatin 3(HTN3).[16] A similar study by Haas et al. (2008) found positive results on stains that were up to 15 months old with higher sensitivity than tests for amylase.[2,17] In another study by Akutsu et al., ELISA for statherin showed higher specificity for detection and was successfully detected in aged saliva stains, mixed body fluids–saliva stains, and simulated casework samples. ELISA for α-amylase showed higher sensitivity than ELISA for statherin, although it was not specific enough to identify saliva.[18]

Samples that test positive in the presumptive tests are subject to confirmatory tests. These investigations include Lateral Flow Immunochromatograhic Strip Test or Rapid Stain Identification (RSID)™ SALIVA Laboratory kits, which test for the alpha amylase molecule and not for the alpha amylase activity. The RSID-saliva test uses a mobile and stationary monoclonal anti-human salivary α-amylase antibody that forms a visible pink line in the presence of antigen. The RSID-saliva test can detect saliva diluted up to 1:10,000, in samples taken from buccal swabs, plastic bottles, plastic mugs, ceramic mugs, cigarette butts, and soda cans.[2] Cross-reaction with blood, semen, urine, vaginal secretions, or menstrual blood was absent.[19–21] Species specificity was also highest with the RSID-saliva test with only rat saliva testing positive.[22]

Raman spectroscopy is a newer non-destructive technique with a great potential for confirmatory identification of body fluids at a crime scene. It has higher selectivity and specificity to chemical and biochemical species with a potential to resolve mixtures of multiple body fluids, with lower sample requirement (picograms or femtoliters) and minimal preparation. Analysis of dry traces of blood, semen, saliva, vaginal fluid, and sweat with Raman microspectroscopy utilizing non-actinic near-infrared light showed that the Raman spectra for each body fluid was unique (Fig. 1). Raman spectroscopy also has the potential to determine the identities of multiple body fluids in a mixture, although more research is needed in this area.[23–25]

2.2. Oral fluid sample collection

The objective of using OF as a source is to analyze DNA from desquamated epithelial cells and leukocytes,[7] as they are obtained using self-administered, noninvasive, and relatively inexpensive techniques.[26] However, OF as a biospecimen suffers from several

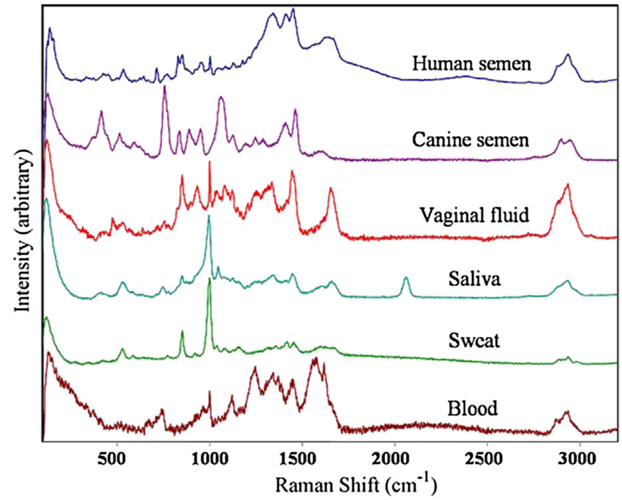

Fig. 1. Raman spectra of human semen, canine semen, vaginal fluid, saliva, sweat, and blood with 785-nm excitation. This is a non-destructive and highly specific technique that yields a unique spectrum for each fluid. The peaks on each spectrum correspond to the characteristic composition of that body fluid, and there is great potential for the analysis for mixtures. (Taken from: Kelly Virkler, Igor K. Lednev. Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Forensic Sci Int **188** (2009) 1–17).

special issues. Depending on the method of collection, the specimen may yield different volumes, affecting the quantity of DNA extracted. Similarly certain compounds in the OF degrade proteins and nucleic acids, when stored and transported at room temperature thus influzening the DNA extracted. Presence of microbial DNA also leads to overestimation of DNA quality and quantity.[27] Furthermore, adherence of cells to sample collection devices (e.g., cotton, foam, and hydrocellulose), decreases the quantity of DNA in the extracted OF specimen.[12]

2.2.1. Recovery of salivary DNA

OF samples are collected directly from the mouth or recovered after deposition on the skin (through biting, sucking, licking) and other inanimate objects such as envelopes, postage stamps, cigarette ends, textiles, soil etc.[28] The quality of the saliva traces present is of interest, since it reveals the blood type of the individual, when the person is a secretor (presence of ABH antigens in body fluids).[29]

2.2.2. Saliva sampling from oral swabs

A fresh OF sample is obtained either by collecting whole saliva or by swabbing the buccal mucosa with Dacron-coated tips, foam-coated tips or tongue depressors.[7] Currently available commercial kits show an increased efficiency of buccal cell collection, storage and isolation of PCR ready DNA e.g. Catch-All™ Sample Collection Swabs, use of Isohelix Dri-Capsules with Isohelix SK-1 swabs.[30] Cytobrushes facilitate the extraction of basal and suprabasal cells with a higher nuclear content, but the percentage of total human DNA obtained is small, with concomitant variation in high molecular weight DNA (>23 kb), suggestive of bacterial contamination.[7,26]

These OF samples are applied onto treated cards such as IsoCode Cards® or the FTA® (Fast Technology for Analysis of nucleic acids) cards (Whatman, GE Healthcare, Florham Park, NJ, USA)[31] for long-term room temperature storage before molecular analyses. These cards have denaturants that protect DNA against oxidation, nuclease and ultraviolet damage; and bacterial/fungal degradation.[7]

2.2.3. Saliva sampling from whole saliva

Whole saliva is preferably collected by swishing with alcohol containing mouthwash and spitting into a collecting vessel. This reduces the bacterial growth during storage and transport.[26]

Commercial saliva collection kits (Oragene® DNA kit) provide an all-in-one system for the collection, stabilization and purification of DNA from OF. This makes the sample suitable for long-term storage at room temperature or in low-temperature freezers. The majority of DNA obtained with Oragene is >23 kb in fragment size and has minimal bacterial DNA (average only 11.8% bacteria).[9]

Rogers et al. compared the DNA yield and quality obtained using a whole-saliva collection device (Oragene™ DNA collection kit) to those from cytobrush, foam swab, and oral rinse. They reported high-quality DNA yield from whole-saliva kit and the oral-rinse method.[13] In contrast, the cytobrush and swab methods provided significantly lower quality of DNA.[26] Similar results were seen in a study by Mulot et al. where high DNA yields were seen with oral rinse as well as cytobrush, whereas DNA yield was lower with treated cards.[32,33]

Studies show that swabs/cytobrushes contain only 11% of human DNA, while mouthwash samples contain 34% to 49% of human DNA. In contrast, whole saliva samples contain an average human DNA yield of 68%. These data corroborate that whole saliva samples are superior to buccal swab samples in the quality of DNA.[34,35]

2.2.4. Saliva sampling from bite marks and other objects

Saliva traces on the skin are obtained using either a single swab with a sterile wet tissue/filter paper placed on the skin or by a double swab technique.[36] In the double swab technique proposed by Sweet, the skin is first gently rubbed with a wet sterile swab, whereby a rehydration and the release of the epithelial cells from dried deposit occurs. The collection of these cells is done with a second dry swab. Both swabs are then gathered into a single sample.[37] This technique is more successful as it collects DNA sequestered within the oral epithelial cells as a result of the rehydration, not just relying upon pure ‘salivary’ DNA, which is susceptible to degradation by nucleases in saliva, especially if it is on a living victim, as the skin's ambient temperature accelerates the process.[5] This technique was also successful in collecting DNA from a bite mark in a victim who had been in a slow running river for over 5 h.[5,37,38]

OF can be isolated from various sources in the crime scene, for example, postage stamps and envelopes, glasses, cigarettes, straws, food and chewing gum, toothbrushes and dental floss, and dental impressions.[36,39,40]

Saliva traces from non-transportable objects are collected by removing the surface bearing the trace, cutting it off at sufficient distance from the edges of the trace. Saliva traces are also collected by grating off the dry traces of saliva and placing them in a clean paper envelope. Saliva traces in soil and sand are collected as a whole and packaged in such a way that shredding does not occur. Packaging and transportation of saliva traces are usually done in paper bags, not plastic bags.[41]

OF found on objects are also collected by first drying the object at room temperature and later the object is cut into pieces and put into a buffer solution. All these samples are stored in sterile refrigerated containers that are labeled and transported to the lab.[36]

In buffers of DNA extraction kits OF is stable at room temperature for at least 1 year. However before adding such buffers OF should be stored on ice (+4 °C) to prevent microbial growth and to decrease salivary DNAse activity. OF can even be frozen and stored at −20 °C or −80 °C before DNA extraction. Extracted DNA can then be frozen and stored at −20 °C or −80 °C until use.[9]

2.3. DNA isolation

The various DNA extraction methods attempt to purify DNA from unwanted biological (i.e., non DNA components of a tissue), substrate (i.e., cotton swabs, clothes or metal objects) and environmental (i.e., dirt) components. Therefore, for routine casework testing, an ideal DNA extraction procedure should enable to recover high amounts of pure DNA from various kind of samples. If possible, it should also be non-toxic and rapid.[42]

Methods such as Phenol-Chloroform (PC), are efficient to remove PCR inhibitors, but may reduce the amount of recovered DNA.[43–45] Chelex 100, an ion-exchange-based method, is more rapid, less prone to sample–sample contamination and, sometimes, more efficient. Although, it recovers substantial amounts of DNA from cytobrush and swab samples, it is inefficient in the removal of PCR inhibitors from old, degraded tissue samples. A modified Chelex method though time consuming yields more of DNA, hence preferred when little DNA is present in the samples.[13,43,46–48]

In silica-based DNA extraction (e.g. QIAamp spin columns), nucleic acids selectively adsorb on a silica support in the presence of a high concentration of chaotropic salts, with washing away of proteins and contaminants. They are labor-saving and easy to perform; however, short DNA fragments that can't bind to a silica surface, render these methods inappropriate in heavily degraded material.[45]

Organic extractions are successful for all sample types and substrates. However, owing to time consumption and use of hazardous chemicals, restricts them to situations where Chelex® or QIAamp® can't provide optimal results. Such is in the case in degraded saliva stains or low volume swabbed saliva samples (1 and 0.5 μl) containing low amounts of DNA.[49]

Currently, processing of small and medium sample throughput is done through automated DNA purification kits based on magnetic particle technology utilizing magnetic beads, paramagnetic-particles or surface-functionalized paramagnetic-particles. They provide consistent yield and purity with no detectable cross-contamination between samples.

Systems designed specifically for forensic casework, such as, the DNA IQ™ System (Promega) or the PrepFiler™ Automated Forensic DNA Extraction Kit (Life Technologies) enable DNA extractions from very small quantities of material, like a blood stain, saliva or semen stains. They are labor-saving and reduce contamination or human error.[45,50,51] Different research groups report a good DNA recovery from low-yield samples using BioRobot® M48 and EZ1 Workstations from QIAGEN. Both the methods show validation for DNA extraction from a variety of sample types, including but not limited to, fresh, frozen and stored blood samples, buccal swabs, dried blood on textiles, hair samples, semen, vaginal and rectal swabs, swabs from bottles and saliva on stamps. There was no evidence of cross-contamination or indication of PCR inhibitors associated with extracted samples.[52,53] Also, these systems allow the extraction of nucleic acids from up to 12 samples simultaneously, taking about 20–40 min. The kits optimized for this system can extract genomic DNA, cellular RNA, viral or bacterial nucleic acids.[54,55]

2.4. DNA analysis (quantification, amplification and interpretation)

DNA profiling methods analyze either chromosomal/ nuclear DNA (nucDNA) or mitochondrial DNA (mtDNA) depending on the quality and quantity of the OF sample, which reveal either sequence specific or length specific variations in the DNA. Large amounts of nonhuman DNA (up to 90% of the total extracted DNA) in saliva samples necessitates DNA quantification methods that are specific for human DNA.[27] Therefore before DNA amplification, successful

isolation of human DNA from the evidentiary material is done using a human specific RT-PCR approach e.g. [Quantifiler™ (Applied Biosystems)].[42]

Short tandem repeats (STR) are currently the industry standard in DNA analysis for forensic applications. STR loci are found throughout the genome and are further distinguished by their location on Y or autosomal chromosome. STRs have high values of polymorphic informativity content (PIC) with a high power of individual discrimination. They are remarkably conserved among species and are similar especially in first-degree relatives. Hence, this technique is especially suited for identification of suspects, victims, and human remains.[45]

STRs found on the Y-chromosome (Y-STRs) pass unchanged through generations, offering distant intergenerational comparisons. These are useful in deficiency paternity cases with male offspring and in forensic genetic cases where the analysis of autosomal STRs failed to give clear conclusions.[56] Also, in a large proportion of mixed male/female stains, the male profile can only be detected through Y-STR analysis, as in sexual assault cases with a male assailant.[13]

Developed countries have maintained a national DNA database, and the STR procedure has simplified the work of forensic and other scientists in DNA-based identification.[7,57] The Federal Bureau of Investigation (FBI) uses a set of 13 specific STR loci collectively referred to as CODIS (Combined DNA Index System) markers, which allows for international standardization of testing procedures. In most kits, full profiles of these independently segregating STR markers have a high power of discrimination (1 in 10^{10} to 1 in 10^{20}).[45,56]

Single Nucleotide Polymorphism (SNP) markers are another important extension to a routine STR-based DNA profiling. SNPs, with a low mutation rate, are stable genetic markers. Differences in SNP markers' distribution and frequency reflect more recent demographic histories (migrations, population bottlenecks, isolation, admixture etc.), and help in inferring the geographic or ethnic origin of an individual. Therefore, SNPs are suitable in paternity testing, analysis using high throughput technologies for data basing, automation and analysis of degraded samples.[58–60] Paternity testing, however is simple and cost efficient using STRs where the experience has accumulated over the last decade.[45]

The enzymes and bacteria in OF render OF stains that are sometimes degraded or yield insufficient quantities of DNA to enable a successful autosomal STR analysis.[61,62] In such cases, mtDNA is a much more convenient marker, due to its high resistance to degradation and a high copy number per cell.[45,63] Lacking in recombination, mtDNA is analyzed as a haplotype. mtDNA shows maternal mode of inheritance, and hence any maternal relative can serve as a reference for personal identification.[45] However, low power of discrimination and deficient database quality, limit the utility of mtDNA in forensic identification. SNPs in the control region outside hypervariable regions I and II (HVI/HVII), and in the coding region of the mtDNA genome, can provide additional discrimination in mtDNA testing.[64]

Conventional polyacrylamide gel electrophoresis (PAGE) methods such as silver staining and fluorescence separate the PCR products generated during AmpFLP. Currently used fluorescence detection methods involve gel or capillary electrophoresis and ABI gel-based DNA sequencers.[55]

A variety of commercial kits are available for robust multiplex amplification of the core STR loci, for e.g. PowerPlex 16 kit (Promega Corporation), 16plex AmpFSTR Identifiler kit (Life Technologies). Standard multiplexes like AmpFlSTR Profiler Plus™ are sensitive, with an optimal input amount of 1 ng of DNA.[42] The analysis of Y-STRs often complement that of autosomal STR markers.[45]

The information obtained after separating the amplified DNA products, needs conversion into a common language that is standard from laboratory to laboratory. Software programs like GeneScan®, Genotyper® software and GeneMapper*ID*® software provide the means to perform the necessary data analysis and standardize the output.[65,66]

3. OF and age estimation

Despite several influences, which contribute to the ageing of mammals, the role of DNA is pivotal.[67] The natural stochastic process of ageing leads to alterations of biomolecules on the molecular level over a lifetime. Age-dependent accumulation of the 4977 bp deletion of mitochondrial DNA and the attrition of telomeres with ageing are two important processes at the DNA-level that are under consideration for age estimation.[68] At present a saliva test based on epigenetic markers is being explored for age estimation.[69]

Aging is a developmentally regulated process, tightly controlled by specific epigenetic modifications. Also, epigenetic factors play an important role in developmental plasticity.[70] The most widely studied epigenetic marker is DNA cytosine methylation, often investigated in the context of CpG dinculeotides in promoter regions (CpG islands).[71] A large number of individual CpG sites have shown a strong linear association with chronological age and DNA-methylation levels.[72] By measuring the methylation level at just two of these spots, researchers at UCLA have estimated the age of an individual to an accuracy of within 5.2 years.[73]

Bocklandt et al. at UCLA described a predictor of age for saliva samples which was generated by a dataset of 34 male twin pairs. Based on three CpG sites associated with the genes, neuronal pentraxin II (NPTX2), EDAR-associated death domain (EDARADD) and target of myb1 (chicken)-like 1 (TOM1L1) they were able to predict donor age in independent saliva samples. Overall, age-associated DNA-methylation changes are highly reproducible but most of them seem to resemble a tissue-specific phenomenon.[73] After analyzing genomes from saliva samples of 34 pairs of identical male twins aged 21–55, they were able to identify 88 sites on the DNA that suggested a high correlation between methylation and age. The study when performed in a random group of 61 men and women aged 18 to 70, created a predictive model by observing just two of the three genes with the strongest age link to methylation. Later both groups' saliva samples were analyzed. Using this predictive model, the team was able to accurately predict each person's age within five years.[74]

In forensic science, such a model is replete with potential applications as it could estimate the age of a person, living or dead, based on a biological sample alone.[75,76]

4. Conclusion and future perspective

OF is gaining prominence as an alternative biological matrix to serum in forensic investigations. In crime scene and related legal investigations it has become instrumental for DNA-based sample collection, owing to lower overall cost, lower infection risk, increased patient convenience, acceptability, compliance and uptake, especially so for children, patients with mental health disabilities, and those who cannot access a blood clinic. For professionals, OF collection carries lesser health risk. Still, a major barrier to using OF in forensics is that it may contain significant proportions of DNA from oral bacteria and/or food. With new and highly sensitive technologies, to recognize human DNA in OF is no longer a limitation.[77]

From the futuristic point of view, the dynamics of modern technical advancements are fast turning OF based diagnostics into a clinical and commercial reality. Miniaturization technology, which

combines microfluidics with micro/nano-electrical-mechanical systems (MEMS & NEMS), has helped create the lab-on-chip (LOC) or micro total analysis system (μTAS).[78,79] This technology has aided the development of miniaturized PCR chips. Such devices allow for shorter assay time, and carry greater potential of integrating multiple processing modules to reduce size and power consumption. The PCR chips are practical in any field where minute amounts of nucleic acid sample require quick amplification and analysis.[79] Now, disposable microfluidic device for on-chip lysing, PCR, and analysis in one continuous-flow process is available for sex determination with human saliva, in less than 20 min from spit to finish.[80,81]

Thus in the post genomic era, the study and use of OF based diagnostics has increased exponentially, as has its acceptance by clinicians and patients. OF therefore, is an ideal translational research tool and diagnostic matrix with novel ways of utilization for forensic investigations. However, additional research coupled with well-controlled study design using neoteric technologies needs to be done to realize the true potential of OF based DNA diagnostics.

Conflict of interest

None declared.

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Ethical approval

None.

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